DNA replication: **Building the perfect switch**

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A sophisticated molecular switch ensures that replication origins are activated just once in each cell cycle. Recent work reveals how the proteolysis of a key replication inhibitor, geminin, by the anaphase promoting complex/cyclosome is an important component of this switch.

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The sequencing of the human genome has been hailed as one of humankind's great achievements, in part, because of the sheer magnitude of the endeavour. The accurate sequence of the three billion or so nucleotides of the human genome has involved many scientists and has taken years to assemble. Yet, every proliferating human cell is faced with the prospect of having to copy accurately and precisely this same information in the space of only a few hours during the cell cycle. Either incomplete replication or over-replication would cause cell death, or worse, would generate the kinds of genetic alterations associated with diseases like cancer.

To accomplish this feat in the allotted time, eukaryotic cells have developed a 'divide and conquer' strategy. Unlike their prokaryotic counterparts, eukaryotic genomes are replicated from multiple replication origins distributed along their chromosomes. In human somatic cells, replication occurs from 10,000–100,000 such replication origins; thus, each replication origin is only responsible for the replication of a relatively small portion of the genome.

This strategy can allow rapid replication of large genomes but brings with it a serious bookkeeping problem. How can the cell keep track of all of these origins, ensuring that each one fires efficiently during S phase while also ensuring that no origin fires more than once? To cope with this, eukaryotic cells have evolved a remarkable molecular switch which, when turned on, promotes just a single initiation event from each origin. Two recent studies [1,2] of DNA replication in *Xenopus* show in greater detail the workings of this switch.

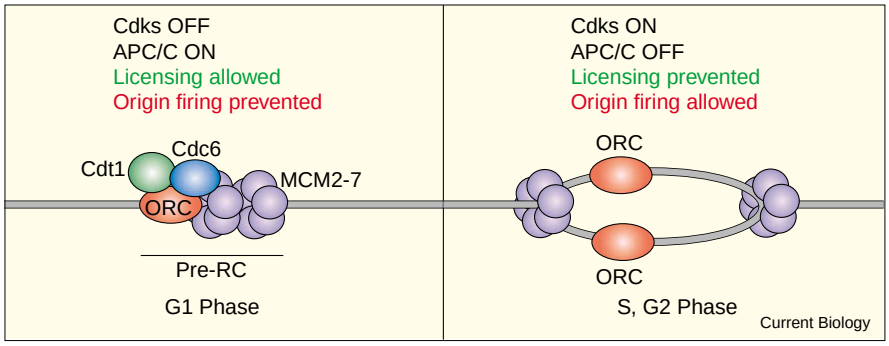
At its heart is the tightly regulated assembly of pre-replicative complexes (pre-RCs) at replication origins in a reaction known as 'licensing' (Figure 1). Pre-RCs assemble in a stepwise manner: the origin recognition complex (ORC), a sequence-specific DNA binding protein, binds first and remains bound to origins during most or all of the cell cycle. Cdc6 then enters the complex, and cooperates with ORC to load six different but related polypeptides known as the Mcm2-7 complex [3]. Recent work in fission yeast and *Xenopus* has identified another pre-RC component, Cdt1, which enters the pre-RC independently of Cdc6 and is also required to load the Mcm2-7 complex [4,5]. The Mcm2-7 complex then plays a crucial role during initiation and during the ensuing elongation phase of DNA replication, perhaps acting as a replicative DNA helicase [6].

Pre-RCs can only assemble at origins during a short period of the cell cycle between the end of mitosis and a point late in G1 phase (Figure 1). This temporal separation of pre-RC assembly and origin activation is a key feature of the switch because it ensures that new pre-RCs cannot assemble on origins which have fired and, thus, origins can fire just once in each cell cycle [7]. Understanding how licensing is prevented after S phase begins, therefore, has been the focus of much research in the field.

Cyclin-dependent kinases (Cdks) are central to this regulation. Cdks are essential for triggering the initiation of DNA replication from origins that contain pre-RCs. At the same time, Cdks appear to play a direct role in preventing the assembly of new pre-RCs [7]. Because Cdk activity remains high from the onset of S phase until the end of the following mitosis, re-licensing of origins cannot occur until the beginning of the next cell cycle. Although the picture is far from complete, it appears that Cdks prevent pre-RC assembly in multiple, redundant ways. In budding yeast, for example, Cdks target Cdc6 for SCF-dependent, ubiquitin-mediated degradation [8–10] and trigger the export of the Mcm2-7 complex from the nucleus [11,12]. It is likely that Cdks also act in other ways to prevent re-replication.

Recent work in *Xenopus* [1,2,13,14] has revealed that another key cell cycle regulator, the anaphase promoting complex/cyclosome (APC/C), plays an important role in constraining licensing to the Cdk cycle. The APC/C is an E3 ubiquitin ligase whose activity is regulated by Cdks: it is activated in mitosis by Cdks associated with mitotic cyclins and inactivated in late G1 phase by Cdks associated with G1 cyclins [13]. In a screen for novel APC/C substrates, McGarry and Kirschner identified a protein they called 'geminin' [14]. Consistent with it being an APC/C substrate, geminin is degraded in mitosis and degradation requires a cyclin-like destruction box near its amino-terminus. By using a destruction box mutant that cannot be degraded, these authors showed that

Figure 1



Regulation of licensing in eukaryotic cells. At the end of mitosis, the anaphase promoting complex/cyclosome (APC/C) is activated by Cdks. Active APC/C then contributes to the inactivation of Cdks by targeting the essential cyclin subunits for ubiquitin mediated degradation. In this state – CDKs off, APC/C on – cells are competent to assemble pre-RCs at their origins. However, the presence of active APC/C prevents accumulation of necessary S phase promoting factors like S phase cyclins and the Cdc7 regulatory subunit, Dbf4. At the end of G1 phase, a switch is thrown which converts cells to a very different state in which origin firing is promoted while licensing is prevented – Cdks on, APC/C off. Both the activation of origin firing and the prevention of licensing requires the activation of Cdks, which in turn requires Cdk-dependent inactivation of the APC/C. Further details are provided in the text.

geminin could inhibit DNA replication and that this inhibition of replication correlated with an inhibition of Mcm loading.

Two groups have recently identified Cdt1 as the target of licensing inhibition by geminin [1,2]. Wolfschlegel *et al.* [1] found human Cdt1 as a protein that is tightly associated with geminin in co-immunoprecipitation experiments from human cell extracts. Using cell-free replication extracts from *Xenopus* eggs, they showed that the inhibition of DNA replication by geminin could be overcome by addition of excess Cdt1 suggesting that geminin may act by inhibiting Cdt1.

Biochemical approaches from Blow and colleagues [15] had identified two protein fractions required for licensing, termed RLF-B and RLF-M. RLF-M was previously shown to comprise a heterohexameric complex of the Mcm2-7 proteins [15]. In their recent work, Tada *et al.* [2] show that geminin acts by inhibiting RLF-B. They used a geminin-affinity chromatography to purify RLF-B and showed that RLF-B appears to be identical to Cdt1. Elution of Cdt1 from the geminin affinity column required 4M urea, attesting to the tight interaction between these two proteins.

Although the regulated appearance and disappearance of geminin could be sufficient to explain how replication occurs only once per cell cycle, it is likely that there will be more to the story. McGarry and Kirschner [14] showed that geminin-depleted *Xenopus* extracts, while supporting efficient DNA replication, did not re-replicate their DNA. This demonstrates that there must be something in these extracts besides geminin which can block re-initiation.

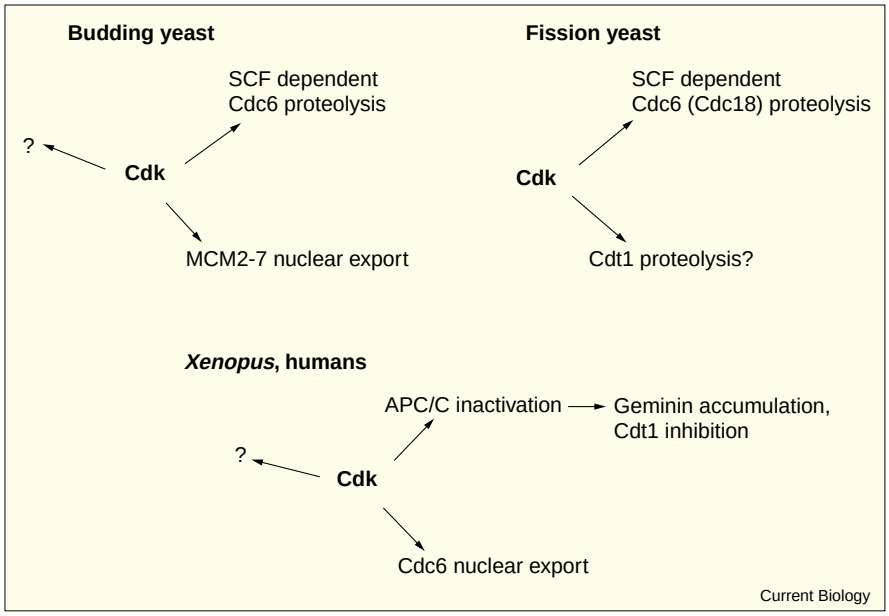
A clue to the nature of this inhibitor may come from the experiments of Tada *et al.* [2] who showed that the partial licensing activity in geminin-depleted metaphase extracts could be significantly enhanced by treatment of extracts with chemical inhibitors of Cdks. This suggests that, even in the absence of geminin, Cdks are able to inhibit licensing to some extent. This is consistent with previous work showing that Cdk2 can prevent licensing in *Xenopus* egg extracts [16].

How does this inhibition work? Experiments in human cells and *Xenopus* [17–19] have shown that phosphorylation of Cdc6, presumably by cyclin A-associated kinase causes its export from nuclei. This may be important for blocking licensing during S and G2 phases, however, it cannot explain the results of Tada *et al.* [2] since licensing in metaphase extracts occurs in the absence of a nuclear envelope. Therefore, there must be some additional and as yet unidentified way in which Cdks can prevent licensing. Thus, in *Xenopus*, as in yeast, Cdks block licensing in multiple, redundant ways. First, they inactivate the APC/C during G1, allowing the accumulation of the licensing inhibitor, geminin. Second, they cause the export of Cdc6 from the nucleus, and third, they act on at least one additional, unidentified target. This may be important in making absolutely sure that re-initiation never occurs and illustrates that such redundancy may be a general feature of the eukaryotic cell cycle.

This points to another emerging trend: Cdks prevent re-replication by different means in different organisms (Figure 2). In budding yeast and fission yeast, Cdc6 — Cdc18 in fission yeast — protein levels are regulated; Cdc6 transcription is limited to late mitosis/early G1 phase and the Cdc6 protein is targeted for ubiquitin-mediated degradation when Cdks are activated in late G1 phase [8–10,20–23]. In *Xenopus*, Cdc6 remains stable during the cell cycle but, instead, Cdk phosphorylation triggers its export from the nucleus [19]. As in *Xenopus*, the nuclear localisation of Cdc6 is also regulated by Cdks in human

Figure 2

Different ways of licensing inhibition in different organisms. Details are provided in the text.



[17,18] and, in addition, Cdc6 is targeted for degradation by the APC/C [24,25].

Cdt1 also appears to be regulated differently in different organisms. Both transcription and proteolysis of Cdt1 are very similar to that of Cdc6 in fission yeast [5]. However, in *Xenopus*, Cdt1 is regulated by geminin which, in turn is targeted for degradation by the APC/C [1,2,12]. In human cells, Cdt1 appears to be regulated by both geminin and, perhaps, cell cycle-regulated proteolysis [1,14]. If a geminin-like licensing inhibitor exists in budding yeast, it is not regulated solely by the APC/C since Cdk inactivation bypasses any requirement for the APC/C in licensing [26]. In a different way, the APC/C may have some role in preventing re-replication in budding yeast, for example, by targeting S phase promoting factors like Clb5 and the Cdc7 regulatory subunit Dbf4 for degradation [27–30].

In conclusion, a single round of DNA replication per cell cycle is achieved by an intricate molecular mechanism which ensures that the 'loading' and 'firing' of replication origins cannot occur in a cell at the same time. The coupling of licensing to the Cdk cycle may be a universal feature of this switch. How this is ultimately achieved may differ in different organisms. Finally, this short review has only addressed the 'negative' side of the switch — how licensing is prevented after S phase begins. The other side of the switch — the mechanism by which Cdks activate replication origins is currently far less well understood and likely to be an area of intense interest in the future.

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